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Inhibition of Biofilm Formation and Pyocyanin Production from Multidrug Resistance *P. aeruginosa* by Using Vitamin C, Salicylic Acid, and Multisera

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Abstract

Pseudomonas aeruginosa is a potent nosocomial pathogen, causing several infections, mostly urinary tract infections (UTIs). The present study is thus aimed to detect the susceptibility pattern of Pseudomonas aeruginosa to antibiotics from urine specimens, and virulence factor production such as (biofilm, and pyocyanin) which are regulated by quorum sensing. The aims were extended to detect the Inhibition of Biofilm formation and pyocyanin production by using vitamin C, salicylic acid and Multi sera. A total of 245 samples were collected from the patients. The samples were subjected to inoculation, isolation and identification of Pseudomonas aeruginosa which were 8 (4%) from total isolates, by standard microbiological procedures. Confirmation of isolates was done by polymerase chain reaction targeting 16srRNA. Antimicrobial sensitivity testing was done using the modified Kirby-Bauer method of the disc diffusion test, with high resistance rates against ceftriaxone (100%) while high sensitivity was to meropenem (88%). Quorum sensing Genes (lasl, lasR) were also detected in Multi-drug resistance and pan resistance isolates. In general, all isolates were Biofilm formation and pyocyanin production. The highest Inhibition of Biofilm was by using vitamin C while the highest Inhibition of pyocyanin was by using salicylic acid.

***Keywords*:** *Biofilm, Multidrug Resistance, Multisera, Pyocyanin, Pseudomonas Aeruginosa, Urinary Tract Infections.*

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative aerobic rod-shaped bacterium that can be isolated from most environments, including soil, plants, and mammal tissue [1]. *P. aeruginosa* can be specified as one of the opportunistic bacteria related to healthcare infections, including ventilator-associated pneumonia (VAP), intensive care unit infections, central line-related bloodstream infections, surgical site infections, urinary tract infections (UTI), burns, wound infections, keratitis, and otitis media [2]. *P. aeruginosa* is an organism capable of adapting to changes in the environment, rapidly developing resistance to antibiotics, and producing a variety of virulence factors. This pathogen can affect immunocompromised patients, in part due to its ability to evade both innate and acquired immune defences through adhesion, colonization, and biofilm formation and to produce various virulence factors that cause significant tissue damage [3]. Biofilm is a complex matrix of extracellular polymeric substances (EPS) that includes glycopeptides, lipids and lipopolysaccharides which protect bacteria from extreme conditions. The matrix allows the inflow of nutrients, water and signalling molecules [4].

Biofilms contribute to the virulence of *P. aeruginosa*, ensuring the colonization of host

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tissues, its immune escape and its resistance to harsh surroundings. The biofilm matrix acts as a natural barrier against the host immune system, preventing bacterial antigen recognition by host antibodies and the penetration of phagocytic cells, such as granulocytes and monocytes. Furthermore, the phagocytosis process is blocked by the very large size of biofilms that appear slightly sensible to enzymatic lysis [5]. *P. aeruginosa* generates a substantial diversity of extracellular pigments, where phenazines are the most important [6]. Pyocyanin is considered a virulence factor for *P. aeruginosa* strains. It can acquire iron from the extracellular environment [7]. It plays a significant role in iron metabolism by actively engaging in reduction mechanisms facilitating the liberation of iron from transferrin [8]. Pyocyanin, the water-soluble pigment, has been shown to exert toxic effects on multiple organs via the production of reactive oxygen species (ROS), Its toxicity arises from its ability to alter the electron transfer pathways, leading to the generation of excess intracellular oxygen reduction products and subsequent cell death. These findings highlight the complex molecular mechanisms underlying the deleterious effects of pyocyanin on cellular physiology and pathogenesis [9].

Quorum sensing (QS) is widely spread in bacteria and is considered as a "speaking" system, QS plays a major role in virulence factor production, and biofilm formation [10]. The QS system regulates various cellular processes, which mainly involve the regulation of bacterial luminescence, virulence factors, disinfectant tolerance, spore formation, toxin production, motility, biofilm formation, and drug resistance [11]. There are three main QS systems in *P. aeruginosa*, two of which use N-acyl homoserine lactones (AHLs) as signal molecules, called the las system, the system, respectively [12] and the third QS system is termed the pqs system, and is interlinked with the other two systems.

Regulation of biofilm formation, Inhibition of QS, and attenuation of virulence factors in *P. aeruginosa* have been reported for a wide variety of extracts, essential oils, and chemical constituents [13]. Pyocyanin or pyoverdine in *P. aeruginosa* may be inhibited by different compounds, such activity was proved for some quaternary ammonium salts, especially those with more than one hydrophilic group [14]. Antibiotics have been widely used to treat biofilm infections, but clinical treatment still faces many challenges due to drug resistance issues, biofilm matrices that hinder drug penetration, and drug-microbe interactions [15].

Materials and Methods

Two hundred forty-five urine samples, clean catch med stream, were collected from UTIs including both sexes with different ages, from different teaching hospitals in Mosul city (Iraq). The study was carried out from March 2023 till September 2023.

P. aeruginosa inoculation, Isolation and identification: The samples were inoculated on the Blood agar plates (Oxoid, UK) and MacConkey agar (Oxoid, UK) incubated at 37 °C overnight aerobically, (Growth which appears non-lactose ferment and oxidase positive), selected and subculture on Pseudomonas cetramide agar (Himedia, India) and incubated in the same condition. Isolates were identified based on their colony morphology, culture, Gram stain, and biochemical characteristics by using an analysis profile index [16].

Molecular diagnosis was done based on 16SrRNA sequencing gene according to [17] and QS Genes (last, last) detection according to [18], Primers Table 1 were designed by the Primer3 program according to the National Center for Biotechnology Information (NCBI).

Table 1. The Sequence of Primers and Their Size

Genes		The sequence of forward and reverse Primer (5′- 3′)	Size (bp)
16SrRNA	F	AGAGTTTGATCMTGGCTCAG	1465
	R	AAGGAGGTGATCCARCCGCA	
las l	F	GGCGCGAAGAGTTCGATAAA	311
	R	TCCAGAGTTGATGGCGAAAC	
las r	F	GATCCTGTTCGGCCTGTT	378
	R	TGCAGTGCGTAGTCCTTG	

Genomic DNA was extracted directly from pure bacterial Growth using a genomic DNA isolation kit supplied by the Gene Aid company. Steps were followed as recommended by the manufacturer. The concentration and purity of genomic DNA were measured by Nanodrop then DNA was stored at -20 °C until further used.

Polymerase chain reaction (PCR) was conducted in a 20 µL volume reaction using GoTaq G2 Green Master Mix (Promega, USA). The universal primers as illustrated in Table 1were used to amplify the full region of the 16S rRNA gene [17]. The concentration of primers (1µM each) and the total amount of template DNA 100ng were added as recommended by the manufacturer. The PCR program for the 16S rRNA gene was set as follows: initial denaturation at 95°C for 3 min followed by 30 cycles of amplification including a denaturation step at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1min. A final extension step was set at 72°C. PCR products were separated on 2% agarose gel and stained with Midori Green Advance DNA stain. A 100bp DNA marker (New England Biolabs, UK) was used as a molecular weight marker.

The antibiotic susceptibility pattern of *P. aeruginosa* clinical isolates was evaluated by the Kirby-Bauer disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) based on the zone of inhibition [19]. The cultures were streaked on Mueller Hinton agar plates and incubated at 37 °C. The zone of inhibition was measured after cultures had been incubated overnight [20]. The test was performed using standard antibiotic discs ceftriaxone (10µg), gentamicin (10µg), ciprofloxacin (10µg), amikacin (10µg), ceftazidime (30µg), meropenem (10µg), piperacillin (100 µg), norfloxacin (30µg), levofloxacin (5µg) (Oxoid, UK).

The total reaction mixture for PCR was 20µl, with 2µl of purified DNA, 10µl of master mix, and 4µl of the primer mix and completing the total volume to 20µl by adding 4µl nuclease-free water [17]. Oligonucleotide primers obtained from HELINI Biomolecules, for lasl, lasR [18], were used as illustrated in Table 1. PCR programming was done with an initial denaturation at 95 °C for 5 min, followed by denaturation, annealing, and extension at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec for 35 cycles. The final extension is at 72 °C for 5 minutes. Gene detection was done on 2% agarose gel, stained

with Midori Green Advance DNA stain. The band pattern was viewed by running electrophoresis at 50V under a trans illuminator for 40 minutes.

Detection of biofilm formation and Pyocyanin production: Biofilm formation determinate according to Sultan *et al*; [20] and Pyocyanin production determinate according to Lahij *et al* [21].

Inhibition of Pyocyanin production and biofilm formation: Three materials Vitamin C, Salicylic acid and Human MULTI-SERA we used to Inhibition. Three concentrations were taken 50µg/ml, 100µg/ml and 150µg/ml for each one Vitamin C and Salicylic acid. MULTI-SERA take 50µL/ml, 100µL/ml and 150µL/ml for Inhibition. Determining the inhibitory action for each material was calculated as a % inhibition using the following equation [22].

$$\% = \frac{AC - AS}{AC} * 100$$

Where, Ac and as are the absorbance of the control and sample, respectively.

Results

The results showed that eight or 4% *P. aeruginosa* were identified from samples based on cultured and biochemical tests. These isolates were found to be negative for lactose fermentation and formed pale yellow colonies on MacConkey agar and produced β-hemolytic on blood agar. The pigments produced on the selective ceramide agar were more obvious (Figure 1).

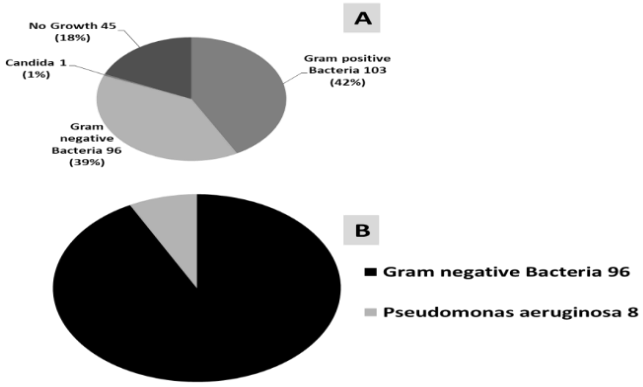

Figure 1. Culture Results of P. Aeruginosa. (A) Culture Results and Percentage, (B) P. Aeruginosa Isolation Percentage

To confirm *P. aeruginosa* isolates, 16SrRNA gene detection was done by using PCR. 16SrRNA genes were purified and sent for sequencing at Psomagene sequencing company (USA). Retrieved sequences were searched for homology against published genes submitted in GenBank using the BLAST tool at NCBI. The 16SrRNA sequencing showed that all isolates (99) % *P. aeruginosa*, were recorded as New four strains in NCBI, Coded (AhmHiy) ACCESSION NO. (OR186518, OR186519, OR186520 and OR186521). All the isolates of *P. aeruginosa* were subjected to antimicrobial susceptibility testing and the highest resistance was found against Ceftriaxone (100 %), while the highest sensitivity was to Meropenem 88%. Among all the isolates, multi-drug-resistant (MDR) *P. aeruginosa* and one isolate were Pan drug-resistant against all antibiotics as shown in Table 2. QS genes (lasl and lasR) were detected in Two Isolates, one MDR and another pan drug resistance, two isolates had lasl and lasR genes. The result of gel electrophoresis for amplification (Figure 2).

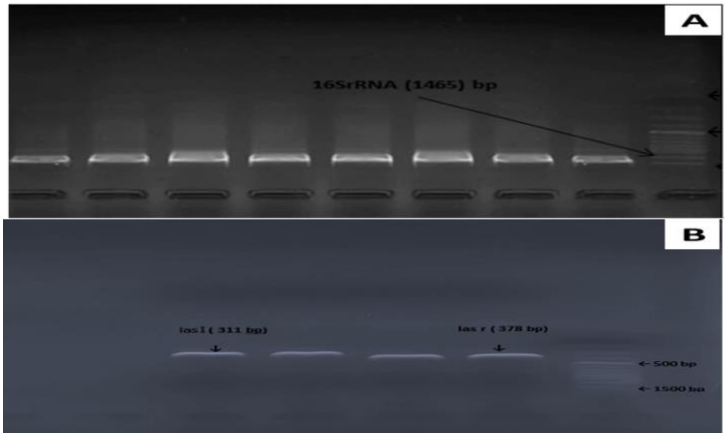

Figure 2. Gel PCR Results. (A) Detection of P. *Aeruginosa* 16SrRNA Gene (1465bp). Lane 1 Shows the DNA Ladder (1500bp) and Lanes 2-9 Represent Positive Results. (B) Electropherogram of the Virulent Gene Determinants with Lane 1 Showing the DNA Ladder (1500bp), Lane 2,3 showing the Positive Amplicons of last (378bp) and Lane 4,5 LasI (311bp)

Table 2. Antibacterial Susceptibility

No. bacterial strain Anti-bacterial	9	23	25	47	72	101	102	170	S%	R%
Amikacin	MS	MS	S	MS	R	R	R	MS	12	38
Gentamicin	MS	S	R	R	S	R	R	MS	25	50
Meropenem	S	S	S	S	S	S	R	S	88	12
Levofloxacin	R	S	S	S	S	S	R	S	75	25
Norfloxacin	R	S	S	S	S	S	R	S	75	25
Ciprofloxacin	R	S	S	S	S	S	R	S	75	25
Ceftazidime	R	R	R	R	R	R	R	S	12	88
Piperacillin	MS	S	R	R	R	S	R	S	38	50
Ceftriaxone	R	R	R	R	R	R	R	R	0	100
S: Sensitive, MS: Moderate Sensitive, R: Resistance										

In the current study, the biofilm formation was determined in Eight Isolates of *P. aeruginosa* by three methods, the results of the Tissue culture plate method (TCPM) were Strong at 75% and Moderate at 25%, the Tube method (TM) appeared that Strong at 63% and Moderate was 37%, and Congo red agar (CRA) did not detected biofilm in all isolates as shown in Table 3. Also, Pyocyanin pigment was estimated by measuring absorbance in Enzyme-linked immunosorbent assay (ELISA) reader equipment (Chromate microplate reader, Awareness Technology INC, USA) at 690nm, the results showed that 8 (100%) of isolates could produce pyocyanin through 3 days of incubation but in varying degrees. Inhibition of biofilm formation and Pyocyanin production was done by using three materials vitamin C, Salicylic acid and Multi sera. Vitamin C was given the highest (Figure 3A). Inhibition for biofilm formation by vitamin C Salicylic acid and Multi sera achieved rates (of 76, 37, 28) % respectively while Salicylic acid was the highest Inhibition for Pyocyanin production then Multi sera and the least of them was vitamin C (77, 56, 38) % respectively (Figure 3B).

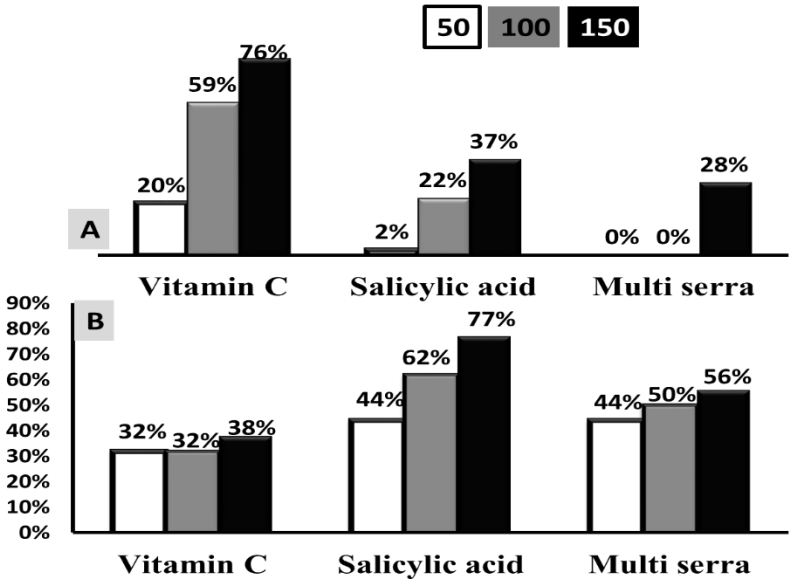

Figure 3. Actions of P. Aerugenosa (A) Inhibition Biofilm Formation (B) Inhibition Pyocyanine Production

Table 3. Grading of Biofilm Formation by Different Methods

Biofilm formation	TCPM	TM	CRA
Strong	6 (75 %)	4 (50 %)	0 (0 %)
Moderate	2 (25%)	3 (37 %)	0 (0 %)
Non/weak	0(0 %)	1 (13%)	8 (100 %)
Total	8 (100 %)	8 (100 %)	8 (100 %)
TCPM=Tissue culture plate method, TM=Tube method, CRA=Congo red agar			

Discussion

Eight strains of *P. aeruginosa* were obtained from 200 positive bacterial cultures of urine samples for the period from March 2023 to September 2023, the percentage of *P. aeruginosa* was 4%, These results were identical to what was obtained by previous studies [23, 24].

The problem of antibiotic resistance is worldwide. *P. aeruginosa* isolates from the current study were multiple antibiotics resistant. *P. aeruginosa* is one of the most important bacterial pathogens seriously contributing to the problem of hospital infections, it's a serious issue that requires urgent attention [25]. The high resistance ratio of *P.aeruginosa* for antibiotics in the current study is due to the indiscriminate use of these antibiotics, in addition to the development of resistance due to the use of sub-therapeutic antibiotic doses which contributed to the emergence of mutant isolates and despite the prevalence of high resistance to antibacterial drugs, This was evident due to the very high resistance to ceftriaxone, as the resistance rate reached 100% [25]. Another study showed that 100 % resistance to ceftriaxone. The same results in the same period for more than one study give a risk indicator of increased bacterial resistance to antibiotics [26]. In the United States, 10–30% of *P. aeruginosa*

isolates were carbapenem-resistant [27]. This percentage is close to our results showed the rate of resistance to meropenem was 12%, This means a high rate of sensitivity to meropenem reaching 88%. By comparing with previous years, we would find that the rate of meropenem sensitivity was greater than it is today, this is due to the same reasons that led to the occurrence of high resistance to ceftriaxone, but at a lower rate, perhaps because the random use of meropenem is less common Among people than other antibiotics. The continuous increase in *P.aeruginosa* resistance to meropenem over time was recorded by Stultz et al [28], which indicated the use of meropenem over 10 years, where the study showed that the rate of meropenem sensitivity to it at the beginning of its use in 2008 was more than 90%, reaching 98% by the year 2011, and they were recorded after that Its sensitivity decreased to about 88% in 2017. The rest of the antibiotics that were used in the current study were distributed between the highest and least effective antibiotics, as shown in Table 2, all antibiotics were chosen based on use treatment (UTIs) and availability in local pharmacies.

The results of QS genes (lasl and lasR) detection showed that MDR isolates were generally expressed all QS genes and these results conducted with Shravani et al. where we used strain No. 102, which was resistant to all antibiotics (PAN), and strain No. 25, which was MDR. This explained a clear picture of possessing these strains and the rest of the QS genes [29]. These genes, in addition to 16SrRNA gen, were also used for accurate diagnosis of *P. aeruginosa* as they are specific genes for this bacterium. In the current study, we used TCPM, TM and CRA methods for phenotypic detection of biofilm formation as shown in Table 3, the highest rate of detection of biofilm production was by TCPM then TM and CRA did not detect, these results were parallel to the results obtained by previous studies [30, 31]. The current study did not agree with a study conducted by Sultan et al [20], where the highest detection rate of biofilm was using the TM. TCPM, that gold standard method, detected biofilm formation. The CRA method showed little correlation with the other methods reported that the CRA method is not appropriate for biofilm detection [32]. In our study, the CRA method does not detect biofilm producers (0 %) and that agreed with the record [30]. The biofilm detection rate using the CRA method was 1.3%. This is a very small percentage compared to the number of bacterial isolates, which amounted to 111.

The differences in biofilm formation with other studies may be due to the variation in culture medium components used in biofilm formation test (e.g. Tryptic Soy Agar or Luria-Bertani broth), the concentration of bacterial cells in culture, or the difference in incubation periods. Also, the cell density in biofilm increases with prolonged length of the incubation period [24]. The concentration of dye used as an indicator in the test could also play a role in the result differences since 5% (w/v) of dye gives better results compared with 1%. The results may also be affected by the type of microplate used in the assay. It was approved that a 96-microplate made of Polystyrene is more efficient in biofilm sticking than a microplate made of other materials such as glass, steel, or granite [25].

Eight isolates of *P. aeruginosa* were subjected to pyocyanin production screening, All the isolates were positive for their ability to produce pyocyanin with varying intensity for the eight isolates. Approximately 90-95% of all *P. aeruginosa* isolates produce pyocyanin as a deep blue referred to as "blue pus" from pyocyanin. Different substances were included in culture media and confirmed the production of high levels of pyocyanin such as glycerol and alanine (as a precursor), Sulphur, and iron [8]. On the other hand, the

differences in the amount of pyocyanin pigment production for our isolates may be due to the presence of regulators of the quorum sensing system named QteE. The overexpression of this regulator will reduce the accumulation of homoserine lactone signals affecting the production of pyocyanin [6].

Inhibition of Pyocyanin production and biofilm formation was conducted using three materials vitamin C, salicylic acid, and Multi sera (paraoxonase containing). Firstly, Ascorbic acid is a reducing agent and rapidly undergoes two consecutive one-electron donation processes to form the ascorbate radical and dehydroascorbic acid [33]. The low concentrations of vitamin C inhibited the expression of several proteins involved directly in the synthesis and export of extracellular polysaccharides. Inhibiting EPS production is a viable strategy for fighting bacterial pathogens. Vitamin C, at low concentrations, can be used to disrupt bacterial biofilm formation by inhibiting EPS production [34]. Pyocyanin structure changed in the presence of ascorbic acid. The changes indicated that ascorbic acid perturbed the pyocyanin aromatic ring, not through the intrinsic acidity of ascorbic acid (as citric acid and dehydroascorbic acid had no effect), but most likely via the intrinsic antioxidant property of ascorbic acid [35].

In *P. aeruginosa*, population-dependent QS is activated through the secretion of AHLs and binding of AHLs to regulatory receptors, initially, LasR and RhlA to promote further biosynthesis of LasR, RhlA and PqsR receptors, which activate the complete QS system activating virulence factor production and biofilm formation [36]. The structure of ascorbic acid (five-membered lactone ring) is analogous to AHLs which leads to competition in binding to the AHL receptor(s) and inhibition of the QS system [37], which leads to Inhibition of Pyocyanin production and biofilm formation This was proven in the current study and the results were consistent with other studies such as [34, 38]. Secondly, Salicylic acid (SA) is the major phenolic acid plant product. The obtained result reported that the addition of SA reduced the biofilm formation in *P. aeruginosa* compared to the untreated control.

Several studies have found that SA can reduce biofilm formation in *P. aeruginosa* [39]. Another study reported that SA suppressed the expression of genes associated with the las/rhl quorum sensing autoinducer 1 (AI1) homoserine lactone-based signalling system in *P. aeruginosa*, Pyocyanin one of the many controlled molecules by the QS [40], we tested the effect of SA on pyocyanin production and the results showed reduction Pyocyanin production compared to the untreated control. Prithiviraj *et al.* indicated that Salicylic acid down-regulates rhlR and lasA in *P. aeruginosa* [41] and causes subsequent inhibition of pyocyanin, protease, and elastase activities [40, 42]. Finally, Multi Sera was used, which is a standard blood serum containing all the substances found in human serum at a high concentration (level 3) from the American company Randox. In the current study, the active substance in the Multi Sera is a paraoxonase.

Human paraoxonase (PON) was originally described based on its ability to degrade organophosphates (OPs), PON enzymes also exhibit lactonase activities, are capable of hydrolyzing and inactivating AHLs, and were used for the disruption of biofilm formation in bacterial pathogens [43, 44]. In the current study, we did not use concentration, but we used volume, due to the difficulty of knowing the concentration of paraoxonase for the Multi Sera, so we used three volumes (50 µ L, 100 µ L, and 150 µ L), there was no inhibition of the biofilm at 50 µ L and 100 µ L and this is due to the low concentration of paraoxonase, but at 150 µ L there was inhibition of biofilm

formation at 28%, while in pyocyanin production the three-volume (50, 100, and 150) μ L resulted in inhibition in Aybey et al. study, respectively [43]. This indicated that pyocyanin is more sensitive to the paraoxonase enzyme than biofilm. Our results agreed with many studies, which indicated that the paraoxonase enzyme had an anti-virulence agent by reducing biofilm formation and pyocyanin production.

It is important to consider the limitations of such a study. Firstly, the effectiveness of vitamin C, salicylic acid, and multi-sera in inhibiting biofilm formation and pyocyanin production may vary depending on the specific strain of Pseudomonas aeruginosa being studied. Different strains may exhibit varying levels of resistance to these agents, which could impact the overall efficacy of the treatment. Additionally, the study should address the potential side effects or adverse reactions associated with the use of these compounds. While vitamin C and salicylic acid are generally considered safe, the concentration and duration of exposure must be carefully regulated to minimize any potential harm to the host. Furthermore, the study should also explore the potential mechanisms by which these compounds interfere with biofilm formation and pyocyanin production. Understanding these underlying mechanisms is crucial for further development and optimization of this treatment approach. Finally, it is important to consider the practical implications of implementing such a treatment strategy. Factors such as cost, availability, and ease of administration should be evaluated to determine the feasibility of using vitamin C, salicylic acid, and multi-sera as potential therapeutic agents.

Conclusion

The *P. aeruginosa* isolates were resistant to most of the tested antibiotics because they produced biofilm and pyocyanin, indicating a correlation between multidrug resistance and the production of biofilm and pyocyanin. All of the isolates were strong biofilm producers except one was moderate. The isolate that gave strong biofilm formation produced a high level of pyocyanin, there is a possibility of inhibiting it using certain materials and concentrations, in the current study we used materials such as antioxidants (vitamin C, salicylic acid) and enzymes (paraoxonase). These materials affected QS, which Naturally controlled for biofilm formation and pyocyanin production This effect may be either by preventing the signal from being received or by destroying the signal itself.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

Not applicable.

References

[1]. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Olson, M. V., 2000, Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, 406(6799), 959-964, doi:10.1038/35023079.

[2]. Ito, C. A. S., Bail, L., Arend, L. N. V. S., Nogueira, K. D. S., Tuon, F. F., 2021, The activity of ceftazidime/avibactam against carbapenem-resistant *Pseudomonas aeruginosa*. *Infectious Diseases*, 53(5), 386-389, doi:10.1080/23744235.2020.1867763.

[3]. Turkina, M. V., Vikström, E., 2019, Bacteria-host crosstalk: sensing of the quorum in the context of *Pseudomonas aeruginosa* infections. *Journal of Innate Immunity*, 11(3), 263-279, doi:10.1159/000494069.

[4]. Alkhulaifi, M. M., 2017, Using Phage's to exterminate biofilms. *Journal of Medical Microbiology and Diagnosis*, 259(6):1-5, doi:10.4172/2161-0703.1000259.

[5]. Duplantier, M., Lohou, E., Sonnet, P., 2021, Quorum sensing inhibitors to quench *P. aeruginosa* pathogenicity. *Pharmaceuticals*, 14(12), 1-35, doi:10.3390/ph14121262.

[6]. El-Fouly, M. Z., Sharaf, A. M., Shahin, A. A. M., El-Bialy, H. A., Omara, A. M. A., 2015, Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. *Journal of Radiation Research and Applied Sciences*, 8(1), 36-48, doi: 10.1016/j.jrras.2014.10.007.

[7]. Wang, T., Sun, W., Fan, L., Hua, C., Wu, N., Fan, S., Yan, J., 2021, An atlas of the binding specificities of transcription factors in *Pseudomonas aeruginosa* directs prediction of novel regulators in virulence. *Elife*, 10, e61885,1-25, doi:10.7554/eLife.61885.

[8]. Jayaseelan, S., Ramaswamy, D., Dharmaraj, S., 2014, Pyocyanin: production, applications, challenges and new insights. *World Journal of Microbiology and Biotechnology*, 30, 1159-1168, doi:10.1007/s11274-013-1552-5.

[9]. Shouman, H., Said, H. S., Kenawy, H. I., Hassan, R., 2023, Molecular and biological characterization of pyocyanin from clinical and environmental *Pseudomonas aeruginosa*. *Microbial Cell Factories*, 22(1), 166, doi:10.1186/s12934-023-02169-0.

[10]. Yin, W. F., Purmal, K., Chin, S., Chan, X. Y., Koh, C. L., Sam, C. K., Chan, K. G., 2012, N-acyl homoserine lactone production by *Klebsiella pneumoniae* isolated from human tongue surface. *Sensors*, 12(3), 3472-3483, doi:10.3390/s120303472.

[11]. Zhao, X., Yu, Z., Ding, T., 2020, Quorum-sensing regulation of antimicrobial resistance in bacteria. *Microorganisms*, 8(3), 1-21, doi:10.3390/microorganisms8030425.

[12]. Chourasiya, S. S., Kathuria, D., Singh, S., Sonawane, V. C., Chakraborti, A. K., Bharatam, P. V., 2015, Design, synthesis and biological evaluation of novel unsymmetrical azines as quorum sensing inhibitors. *RSC advances*, 5(97), 80027-80038, doi:10.1039/C5RA12925G.

[13]. Abdel Bar, F. M., Alossaimi, M. A., Elekhnawy, E., Alzeer, M. A. A., Abo Kamer, A., Moglad, E., ElNaggar, M. H., 2022, Anti-quorum sensing and anti-biofilm activity of Pelargonium× hortorum root extract against *Pseudomonas aeruginosa*: Combinatorial effect of catechin and gallic acid. *Molecules*, 27(22), 7841, doi:10.3390/molecules27227841.

[14]. Piecuch, A., Lamch, Ł., Paluch, E., Obłąk, E., Wilk, K. A., 2016, Biofilm prevention by dicephalic cationic surfactants and their interactions with DNA. *Journal of Applied Microbiology*, 121(3), 682-692, doi:10.1111/jam.13204.

[15]. Kamaruzzaman, N. F., Tan, L. P., Mat Yazid, K. A., Saeed, S. I., Hamdan, R. H., Choong, S. S., Gibson, A. J., 2018, Targeting the bacterial protective armour; challenges and novel strategies in the treatment of microbial biofilm. *Materials*, 11(9),1-27, doi:10.3390/ma11091705.

[16]. Abdulhaq, N., Nawaz, Z., Zahoor, M. A., Siddique, A. B., 2020, Association of biofilm formation with multi drug resistance in clinical isolates of *Pseudomonas aeruginosa*. *EXCLI Journal*, 19(1), 201-208, doi:10.17179/EXCLI2019-2049.

[17]. Khaleel, A. M., Faisal, R. M., Altaii, H. A., 2023, The efficiency of molecular methods compared to traditional methods in identifying bacteria from blood and cerebrospinal fluid samples. *Malaysian Journal of Microbiology*, 19(2):1-10, doi:10.21161/mjm.220105.

[18]. Khalid, I., Nayyef, N. S., Merkhan, M. M., 2022, A Taxonomic Study comparing the two types of Medicinal Leeches available in Iraq. *Research Journal of Pharmacy and Technology*, 15(3), 1119-1122, doi:10.52711/0974-360X.2022.00187.

[19]. Humphries, R., Bobenchik, A. M., Hindler, J. A., Schuetz, A. N., 2021, Overview of changes to the clinical and laboratory

standards institute performance standards for antimicrobial susceptibility testing, M100. *Journal of Clinical Microbiology*, 59(12), 10-1128, doi:10.1128/JCM.00213-21.

[20]. Sultan, A. M., Amer, G. F., Nabiel, Y., 2021, Quinolone-resistant uropathogenic E. coli: is there a relation between qnr genes, gyrA gene target site mutation and biofilm formation? *Journal of Medical Microbiology*, 70(10), 001432, doi:10.1099/jmm.0.001432.

[21]. Lahij, H. F., Alkhater, A. H., Hassan, M. H., Yassir, L. A., 2021, The Effect of Qourum Sensing genes (lasI, rhlI) in Some Virulence Factors of *Pseudomonas aeruginosa* Isolated from Different Clinical Sources. *Medico-legal Update*, 21(1),303-308, doi:10.37506/mlu. v21i1.2325.

[22]. Mansuri, A., Lokhande, K., Kore, S., Gaikwad, S., Nawani, N., Swamy, K. V., Pawar, S., 2022, Antioxidant, anti-quorum sensing, biofilm inhibitory activities and chemical composition of Patchouli essential oil: *in vitro* and *in silico* approach. *Journal of Biomolecular Structure and Dynamics*, 40(1), 154-165, doi:10.1080/07391102.2020.1810124.

[23]. Farhadi, Z., Bahador, N., 2018, Pathotypic and phylogenetic studies of urine *Escherichia coli* isolates from girls< 5 years of age in Marvdasht hospital. *Biomedical and Biotechnology Research Journal (BBRJ)*, 2(4), 281-285, doi: 10.4103/bbrj.bbrj_73_18.

[24]. AL-Khikani, F. H. O., Ayit, A. S., 2019, Correlation study between urinary tract bacterial infection and some acute inflammatory responses. *Biomedical and Biotechnology Research Journal* (BBRJ), 3(4), 236-239, doi: 10.4103/bbrj.bbrj_122_19.

[25]. Kassob, D. S., Hummadi, E. H., 2023, Study of pyocyanin production and biofilm formation in clinical *Pseudomonas aeruginosa*. *Academic Science Journal*, 1(2),139-152, doi:10.24237/ASJ.01.02.648B.

[26]. Abbas, R., Nawaz, Z., Siddique, A. B., Aslam, R., Rafique, A., Zahoor, M. A., Alsayeqh, A. F., 2022, Molecular Detection of Biofilm Production among Multidrug Resistant Isolates of *Pseudomonas aeruginosa* from Meat Samples. *Pakistan Veterinary Journal*, 42(4), 505-510, doi: 10.29261/pakvetj/2022.074.

[27]. Tenover, F. C., Nicolau, D. P., Gill, C. M., 2022, Carbapenemase-producing *Pseudomonas aeruginosa*–an emerging challenge. *Emerging Microbes & Infections*, 11(1), 811-814, doi:10.1080/22221751.2022.2048972.

[28]. Stultz, J. S., Arnold, S. R., Shelton, C. M., Bagga, B., Lee, K. R., 2019, Antimicrobial stewardship impact on *Pseudomonas aeruginosa* susceptibility to meropenem at a tertiary pediatric institution. *American Journal of Infection Control*, 47(12), 1513-1515, doi: 10.1016/j.ajic.2019.05.001.

[29]. Shravani, V., Selvi, G. A. S., Mantravadi, H., 2023, Detection of quorum sensing virulence factor genes and its consanguinity to antibiotic sensitivity profile in the clinical isolates of *Pseudomonas aeruginosa*. *Iranian Journal of Basic Medical Sciences*, 26(8), 899: 899-905, doi:10.22038/ijbms.2023.67981.14992.

[30]. Halim, R. M. A., Kassem, N. N., Mahmoud, B. S., 2018, Detection of biofilm-producing staphylococci among different clinical isolates and its relation to methicillin susceptibility. Open access *Macedonian Journal of Medical Sciences*, 6(8),1335–1341, doi:10.3889/oamjms.2018.246.

[31]. Mohsenzadeh, A., Fazel, A., Bavari, S., Borji, S., Pourasghar, S., Azimi, T., Sabati, H., 2021, Detecting of biofilm formation in the clinical isolates of *Pseudomonas aeruginosa* and Escherichia coli: an evaluation of different screening methods. *Journal of Current Biomedical Reports*, 2(2), 56-61, doi:10.52547/JCBioR.2.2.56.

[32]. Siddhiqui, S., Afreen, U., Kotgire, S., 2018, Evaluation of biofilm formation by three different methods and its antibiogram with special reference to indwelling medical devices from a tertiary care hospital. *Annals of*

Laboratory Medicine, 5(2), 171-76, doi:10.21276/APALM.1630.

[33]. Padayatty, S. J., Levine, M., 2016, Vitamin C: the known and the unknown and Goldilocks. *Oral Diseases*, 22(6), 463-493, doi:10.1111/odi.12446.

[34]. Pandit, S., Ravikumar, V., Abdel-Haleem, A. M., Derouiche, A., Mokkapati, V. R. S. S., Sihlbom, C., Mijakovic, I., 2017, Low concentrations of vitamin C reduce the synthesis of extracellular polymers and destabilize bacterial biofilms. Frontiers in microbiology, 8(1), 1-11, doi:10.3389/fmicb.2017.02599.

[35]. Das, T., Kutty, S. K., Tavallaie, R., Ibugo, A. I., Panchompoo, J., Sehar, S., Manefield, M., 2015, Phenazine virulence factor binding to extracellular DNA is important for *Pseudomonas aeruginosa* biofilm formation. *Scientific Reports*, 5(1):1-9, doi:10.1038/srep08398.

[36]. Lee, J., Zhang, L., 2015, The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein and Cell*, 6(1), 26-41, doi:10.1007/s13238-014-0100-x.

[37]. Han, B., Zheng, X., Baruah, K., Bossier, P., 2020, Sodium ascorbate as a quorum-sensing inhibitor leads to decreased virulence in Vibrio campbellii. *Frontiers in Microbiology*, 11, 1054, doi:10.3389/fmicb.2020.01054.

[38]. Das, T., Das, B., Young, B. C., Aldilla, V., Sabir, S., Almohaywi, B., Kumar, N., 2023, Ascorbic acid modulates the structure of the *Pseudomonas aeruginosa* virulence factor pyocyanin and ascorbic acid-furanone-30 combination facilitate biofilm disruption. *Frontiers in Microbiology*, 14(1),1-15, doi:10.3389/fmicb.2023.1166607.

[39]. Lattab, A., Rachid, D., Arabi, A., Hichem, D., Com, A. Y., 2017, Effect of salicylic acid on biofilm formation and on some virulence factors in *Pseudomonas aeruginosa*. *International Journal of Biosciences*, 10, 60-71, doi:10.12692/ijb/10.1.60-71.

[40]. Yang, L., Rybtke, M. T., Jakobsen, T. H., Hentzer, M., Bjarnsholt, T., Givskov, M., Tolker-Nielsen, T., 2009, Computer-aided identification of recognized drugs as *Pseudomonas aeruginosa* quorum-sensing inhibitors. Antimicrobial agents and chemotherapy, 53(6), 2432-2443, doi:10.1128/AAC.01283-08.

[41]. Prithiviraj, B., Bais, H. P., Jha, A. K., Vivanco, J. M., 2005, *Staphylococcus aureus* pathogenicity on *Arabidopsis thaliana* is mediated either by a direct effect of salicylic acid on the pathogen or by SA-dependent, NPR1-independent host responses. *The Plant Journal*, 42(3), 417-432, doi:10.1111/j.1365-313X.2005.02385. x.

[42]. Guo, M., Gamby, S., Zheng, Y., Sintim, H. O., 2013, Small molecule inhibitors of AI-2 signalling in bacteria: state-of-the-art and future perspectives for anti-quorum sensing agents. *International Journal of Molecular Sciences*, 14(9), 17694-17728, doi:10.3390/ijms140917694.

[43]. Aybey, A., Demirkan, E., 2016, Inhibition of *Pseudomonas aeruginosa* biofilm formation and motilities by human serum paraoxonase (hPON1). *AIMS Microbiology*, 2(4), 388-401, doi:10.3934/microbiol.2016.4.388.

[44]. Khatun, M. A., Hoque, M. A., Koffas, M., Feng, Y., 2023, Reducing the virulence of *Pseudomonas aeruginosa* by using multiple quorum-quenching enzymes. *Journal of Industrial Microbiology and Biotechnology*, 50(1), kuad028, doi:10.1093/jimb/kuad028.